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Characterization and Optimization of Auto-Transplantation and Allo-transplantation of Free Composite Tissue for Reconstruction of Battlefield Injuries

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Microvascular porcine model for the optimization of vascularized composite tissue transplantation

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ABSTRACT

Background: Devastating extremity injuries are prevalent but most often survivable on the modern battlefield. The complexity of these injuries requires advanced methods of reconstruction. This study is designed to validate the feasibility of gracilis myocutaneous flap transplantation via microvascular free tissue transfer in a porcine model. This model will facilitate study of autotransplant physiology as well as vascularized composite allotransplantation as an evolving method for reconstructing previously nonreconstructable injuries.

Material and methods: A donor gracilis myocutaneous flap is procured from Yorkshire swine. The right external carotid artery and internal jugular vein are prepared as the recipient axis for microvascular anastomoses. Group 1 undergoes immediate microvascular anastomosis with resultant 1-h ischemic period. Group 2 undergoes delayed anastomosis with 3-h ischemic period. Markers of ischemia-reperfusion injury are evaluated after anastomosis and on postoperative days 1, 2, 7, and 14.

Results: A novel porcine model for microvascular composite tissue transplantation is demonstrated. Ischemia period-dependent elevations in circulating biomarkers (lactate dehydrogenase [LDH], creatine kinase [CK], and aspartate transaminase [AST]) demonstrate the effects of prolonged ischemia. Both groups showed marked LDH elevation without significant statistical intergroup difference ($P = 0.250$). The difference in CK and AST levels at 24 h showed strong significance ($P < 0.0001$).

Conclusions: A novel method of vascularized gracilis myocutaneous flap transplantation was validated in the Yorkshire swine. Assays for skeletal muscle tissue injury (LDH, CK, and AST) showed ischemia period-dependent response providing assessment of ischemia-reperfusion injury at the cellular level. Subsequent studies will evaluate agents that mitigate ischemia-reperfusion injury and transition these findings to potentiate vascularized composite allotransplantation.

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1. Introduction

Efforts to ameliorate the lethal impact of ballistic and improvised explosive devices have resulted in the development of modern body armor, which effectively shields the torso but leaves the extremities and maxillofacial regions vulnerable [1]. In combination with rapid evacuation times and advanced critical care, modern body armor has reduced mortality rates in current conflicts of Operation Iraqi Freedom/Operation Enduring Freedom (OIF/OEF). Troops that would have otherwise suffered fatal injuries now present with more complex extremity and facial wounding patterns, which require complex reconstruction [2,3]. Combat-related extremity and maxillofacial trauma result in substantial functional morbidity and limb loss in deployed service members [4]. Both military and civilian demands for extensive complex vascularized composite tissue reconstruction require improvements in the current techniques. Optimization of physiologic parameters during vascularized composite tissue transfer will enhance current techniques and offer significant advantages to the field of reconstructive allotransplantation.

The objectives of this study are to validate the feasibility of gracilis myocutaneous flap autotransplantation and allotransplantation via the use of microvascular free tissue transfer in a porcine model. This model will be used to demonstrate the impact of immediate and delayed restoration of blood flow after flap harvest. After tissue reperfusion, systemic markers of ischemic injury (lactate dehydrogenase [LDH], creatine kinase [CK], and aspartate transaminase [AST]) will be assayed throughout the 14-d survival period to assess the impact of ischemia-reperfusion injury on composite tissue viability [5–7].

The porcine large animal model was chosen as a close analog to human vascular anatomy and physiology. Gracilis myocutaneous flap anatomy is similar to the human in location, vascular pedicle, and innervation [8]. The donor site defect represents minor morbidity as evidenced by typical return of normal gait at 2 d after transfer. The recipient site vascular anatomy of the external carotid artery and internal jugular vein is highly preserved and easily accessible. The neck recipient site was chosen for its reliability, safety, and limited morbidity.

This model will be used in future studies to develop advanced methods of tissue stabilization to improve tissue viability and success rates in vascularized composite tissue autotransplantation. As a natural continuation, this model will facilitate study of vascularized composite allotransplantation as an evolving method for reconstructing previously nonreconstructable injuries. Ultimately, this large animal platform will serve as a mechanism for investigation of allograft immunotolerance induction.

2. Materials and methods

Adult female Yorkshire swine (John Albert, Cibolo, TX) weighing 70–90 kg were used for all experiments. Animals were handled and cared for under institutional guidelines in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC: National Academy Press, 1996) and the American Association for the Accreditation of Laboratory Animal Care. Animals were entered into the study after a 5-d period of acclimation in the housing facilities of the Wilford Hall Ambulatory Surgical Center Clinical Investigations Division. All animals were inspected before the study by a veterinarian and monitored daily by a technician. The experimental protocols were approved by the Wilford Hall Ambulatory Surgical Center Clinical Investigations Division Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of Good Laboratory Practices.

All animals were sedated 30–45 min before induction of anesthesia with ketamine (15–20 mg/kg) (Fort Dodge Ketaset, manufactured for Fort Dodge Animal Health, Fort Dodge, Iowa) and atropine (0.04–0.4 mg/kg) (Baxter International Inc., Deerfield, Illinois) administered intramuscularly by veterinary technicians. The animals were pre-oxygenated for 2–3 min by mask with 100% ambient oxygen. Anesthesia was then initiated with isoflurane 3%–4.5% in an air-oxygen mixture of 40%–60%. The animals were then orally intubated with an appropriate size (6.0–7.5 mm) high-volume, low-pressure cuff endotracheal tube. Bilateral breath sounds were checked after intubation and transport. Anesthesia was maintained with isoflurane 2%–3% in an air-oxygen mixture of 40%–60%, by the nurse anesthetist or the qualified surgical technician. During the period of general anesthesia, the animal's mean arterial blood pressure, heart rate, respiratory rate, O_2 saturation, and CO_2 levels were continuously monitored.

2.1. *Gracilis myocutaneous flap dissection*

After induction of general anesthesia, the swine is placed in the supine position. Baseline biomarkers (Complete Blood Count (CBC), chemistry, pH, pO2, pCO2, HCO3, CK, lactic acid, LDH, and AST) are obtained before incision. Both hind limbs are retracted cephalad and secured in place. The left hind limb is prepped and draped in sterile fashion. To establish reliable landmarks for flap design, a line is first drawn along the posteromedial thigh from the vaginal orifice to the hock of the left hind limb (Figs. 1 and 2). Next, a bisecting line at 20° to the original line is drawn; this designates the course of the gracilis muscle. The intersection of the lines defines the medial extent of the skin island. A 6.0 × 3.5 cm ellipse is then drawn centered immediately lateral to the intersecting lines. The medial border of the ellipse is then incised through the skin and subcutaneous tissue to the level of the muscular fascia taking care to preserve musculocutaneous perforators. The muscular fascia is then incised, and the gracilis muscle is visualized (Fig. 3). After ensuring a centralized position of the skin island over the gracilis muscle, the remainder of the ellipse is then incised. Dissecting deep and anterior to the gracilis muscle reveals the anterior branch of the obturator nerve and the dominant vascular pedicle (medial circumflex femoral artery and veins) (Fig. 4). The vessels are dissected and noncontributing

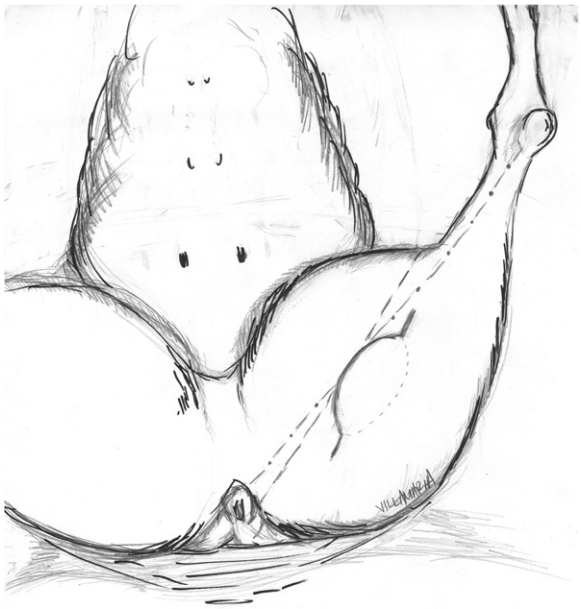

Fig. 1 – Depiction of landmarks for preoperative markings.

branches ligated. Once the vessels are dissected free from surrounding structures, the gracilis muscle is sharply divided proximally and distally. The vessels of the exposed pedicle are ligated proximally and divided. The composite tissue flap containing muscle, fascia, subcutaneous tissue, skin, and vessels is removed. Flap weight is 400 ± 20 gm. Pedicle length is typically 3–4 cm. The flap is then flushed in antegrade fashion with heparinized saline solution (10 u/cc) while awaiting transplant into the neck. Flaps then undergo either immediate or delayed inset and reperfusion (1 h [$n = 6$] or 3 h [$n = 6$] of ischemia, respectively). Flaps designated to undergo 3 h of ischemia are kept at 4°C during the idle portion of the ischemic period. The hind limb defect is copiously irrigated with saline. Hemostasis is ensured and the defect is closed in layers over a 1" Penrose drain with interrupted 3.0-monocryl subcutaneous sutures followed by full thickness 0-prolene horizontal mattress sutures.

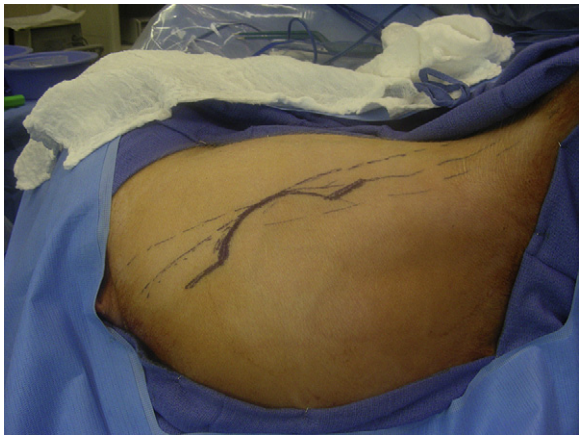

Fig. 2 – Preoperative markings.

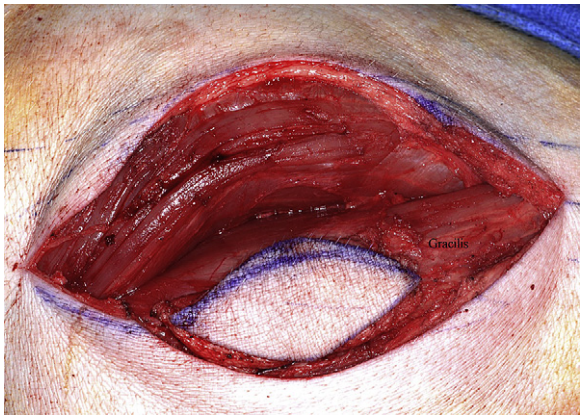

Fig. 3 – Defined skin island centralized over gracilis muscle belly.

2.2. *Neck exposure and dissection*

In the supine position, the neck is prepped and draped in sterile fashion from sternal notch to the angle of the mandible. A 12 cm longitudinal incision is made 2 cm to the right of midline from below the angle of the mandible to the sternal notch (Fig. 5). The sternocleidomastoid muscle is retracted laterally and dissection is continued deep until the external carotid artery and internal jugular vein are exposed (Fig. 6). The vessels are dissected free from surrounding structures for a length of approximately 5 cm to provide adequate mobility for anastomosis.

2.3. *Gracilis myocutaneous flap microvascular transfer*

After exposure of neck vessels and procurement of the gracilis flap, the flap is prepared for anastomosis within the neck cavity. Using the right external carotid artery and internal jugular vein as the recipient axis, microvascular anastomosis is performed. The surgical microscope Leica M535 F20 (Leica Microsystems, Wetzlar, Germany) is positioned for optimal view of the neck cavity. With a proximally placed vascular

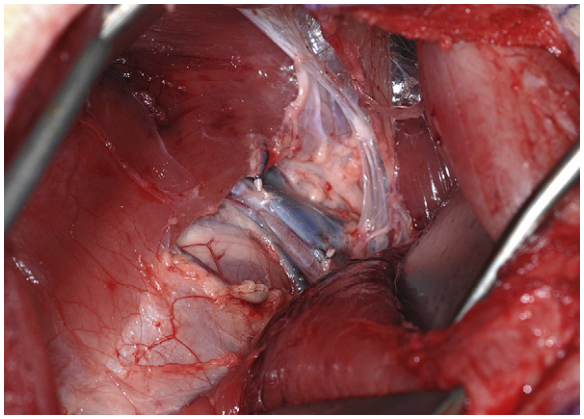

Fig. 4 – Dominant vascular pedicle to the gracilis (medial circumflex femoral artery and vein) deep and medial to gracilis.

Fig. 5 – Depiction of neck incision (dashed line) 12 cm in length positioned 2 cm to the right of the midline.

clamp, the internal jugular vein is ligated and divided. Heparinized saline flush (10,000 units/L) is infused into the proximal end of the vessel. Similarly, the external carotid artery is clamped and flushed retrograde with heparinized saline. The venous anastomosis is performed first with the use of a 2.5 mm venous coupler (Synovis GEM™ FLOW COUPLER®, St. Paul, MN). Once the venous anastomosis is completed, the arterial anastomosis is performed with two running hemicircumferential 8.0 nylon sutures (Fig. 7). The vascular clamps are then removed and perfusion reestablished. The venous FLOW COUPLER® is connected to the external audio box to confirm Doppler signal and patency. Once reperfusion is confirmed, blood is drawn from the recipient vein with a 25 gauge angiocatheter for biomarker assays. The coupler wire remains attached to the external monitor while the neck incision is closed to ensure that there is no positional obstruction of venous flow. The neck incision is closed in layers with interrupted 3.0-monocryl deep dermal sutures followed by a running 3-0 nylon suture Fig. 9a. After the completion of flap inset, the oxygen saturation (rSO_2) of the

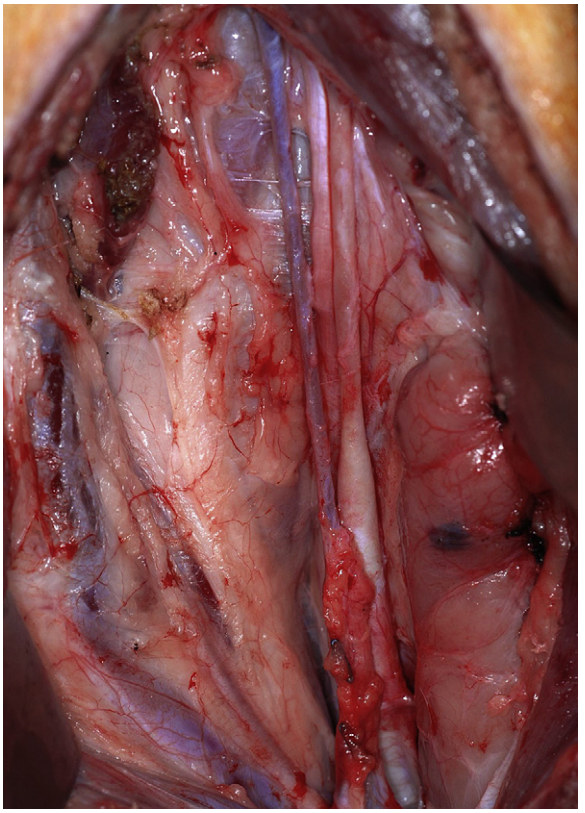

Fig. 6 – Dissection of recipient neck vessels.

flap is assessed using transcutaneous optical spectroscopy (Somanetics INVOS®, Troy, MI).

Anesthesia is then weaned. The animal is extubated and transported to the animal care holding facility. During the postoperative survival period, the animals are housed in individual runs with freedom of movement and positioning.

2.4. *Postoperative assessments*

On postoperative days (POD) 1, 2, 7, and 14 the animals are sedated with ketamine (15–20 mg/kg) and placed supine on the operating table for reassessment. Blood samples are collected and assayed for systemic biomarkers (CBC,

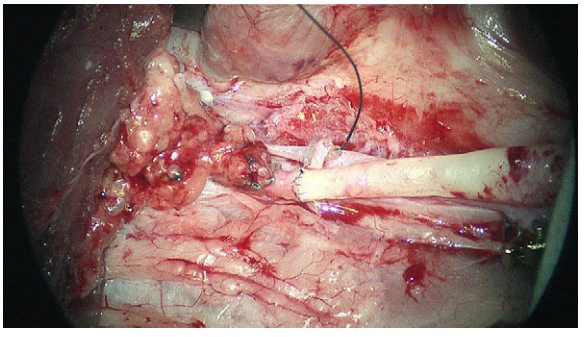

Fig. 7 – Arterial and venous anastomoses.

chemistry, pH, pO2, pCO2, HCO3, CK, lactic acid, LDH, and AST). The flap appearance and incisions are examined. Patency of the venous anastomosis is assessed via external attachment of venous FLOW COUPLER®. O_2 saturation of the flap also is compared with that of the surrounding skin via use of the Somanetics INVOS® device. On POD 14, animals are reassessed and then euthanized. The flap is procured and sent to pathology. In the event of flap nonviability the animal is euthanized and the flap sent for pathologic examination. Six model development and 13 experimental animals were used for this study. Flap failure occurred in two model development animals and one experimental animal.

2.5. *Statistical analysis*

The experimental design of this study was a mixed-effects, randomized complete block design with repeated measures. Subject was a random effect as subjects were a sample randomly selected and randomly assigned to a group. Fixed effects were ischemia period, treatment group, and time of repeated measure as these effects cannot be generalized to other treatments and times.

Sample means and standard errors were calculated for continuous dependent variables and were stratified by group. Frequency distributions, by group, were determined for categorical variables. Main effects (group, time, and group by time) for continuous data were tested (at $\alpha = 0.05$) using a mixed-effects repeated measures multivariate analysis of variance. The Bonferroni method was used to correct the level of significance for multiple comparisons while investigating significant main effects. For categorical variables, frequency distributions by group were compared using Fisher's Exact Test ($\alpha = 0.05$). Analyses were performed using SASR 9.2, SAS Institute, Inc., Cary, NC.

3. Results and discussion

The feasibility of a porcine model of vascularized gracilis myocutaneous flap transplantation was demonstrated. Long-term vascularized composite tissue viability was observed in the setting of immediate (1 h) and delayed (3 h) restoration of blood flow. On POD 14 the donor myocutaneous flaps in both 1-h and 3-h ischemic time groups were well integrated with the surrounding skin (Fig. 9). Flap harvest also revealed incorporation of donor muscle with surrounding recipient tissues.

Assessments of biomarkers on PODs 1, 2, 7, and 14 revealed marked effects of ischemia-reperfusion injury at the cellular level. LDH, CK, and AST were compared as these enzymes are prevalent in skeletal muscle and routinely measured as markers for ischemia-reperfusion injury [5,6].

LDH peaked for both ischemic time groups at 24 h (Fig. 10). Those flaps that were subjected to 1 h of ischemia had a mean LDH level of 24×10^3 U/L ($\pm 5 \times 10^3$) compared with the group subjected to 3 h of ischemia with 31×10^3 U/L ($\pm 6 \times 10^3$). Both groups showed marked LDH elevation, but there was no significant statistical intergroup difference ($P = 0.250$). After levels peaked at POD 1 the LDH levels for both groups asymptotically returned toward baseline through POD 14.

Fig. 11 shows the progression of CK in both ischemic groups. Again there was a notable increase in enzyme levels at 24 h by both groups. The CK level for the 1-h ischemic group was 24×10^3 U/L ($\pm 5 \times 10^3$) compared with a CK level of 75×10^3 U/L ($\pm 14 \times 10^3$) for those subjected to 3 h of ischemia. The intergroup difference in CK levels at 24 h showed strong significance ($P = <0.0001$). Similarly to the LDH group, enzyme levels asymptotically flattened over the 2-wk observation period.

The AST curve mirrors CK levels closely between both groups and also shows strong statistical significance (721 U/L [±72] *versus* 2293 U/L [±421], $P < 0.0001$) (Fig. 8).

Significant ischemia period-dependent elevations in tissue enzyme levels demonstrate the effects of prolonged ischemia and subsequent reperfusion injury to the tissue. These results underscore that cellular level assessment of delayed restoration of blood flow can be evaluated with systemic biomarkers (LDH, CK, and AST) especially within the first 24 h after reperfusion and therefore can be used as markers of the magnitude of tissue injury in this model.

Histological analysis of those flaps that survived the 14 d postoperatively and those that failed before the designated survival period was performed. Fig. 12 illustrates the appearance of normal flap microvasculature at the end of 14 d after autotransplantation (A); this can be compared with the vascular congestion associated with flap loss (B). Comparison of longitudinal sections of muscles between those flaps that survived with those that failed is demonstrated (Fig. 13). Fig. 13A represents the appearance of muscle tissue in a flap that has survived. It contains an increased number of satellite cells. Cross striations are clearly visible, representing a regenerative response. In comparison, Fig. 13B depicts a myocyte that is swollen, with a vacuolated sarcoplasm and no visible cross striations, representing necrosis. Additionally, myocytes are separated by clear space indicative of edema.

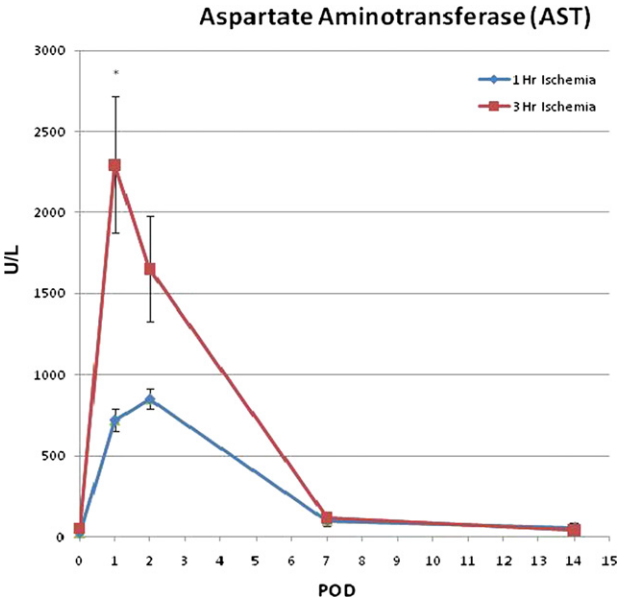

Fig. 8 – AST levels during experimental course. *P < 0.02.

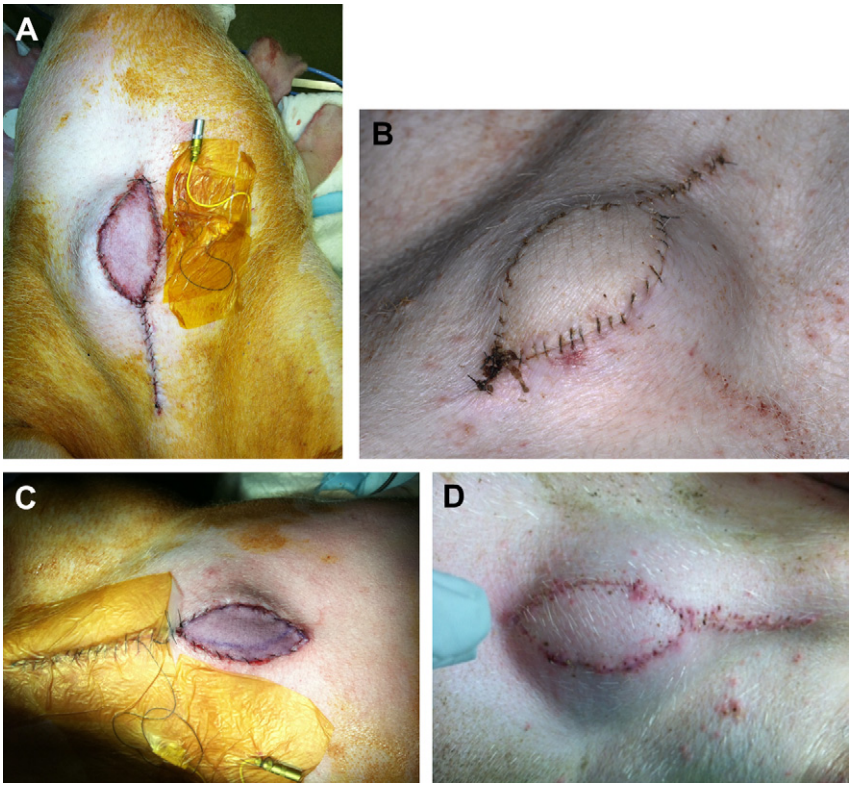

Fig. 9 – Gracilis myocutaneous flap after inset after 1 h of ischemia. (A) Appearance of gracilis flap immediately postoperatively. (B) Flap at POD 14. Gracilis myocutaneous flap after inset after 3 h of ischemia. (C) Gracilis flap immediately postoperatively. (D) Flap at POD 14.

4. Discussion

Devastating extremity injuries are prevalent on the modern battlefield. Improved body armor, rapid evacuation times, and advanced critical care yield survivability in the face of catastrophic limb trauma. The number and complexity of these injuries require advanced methods for reconstruction and salvage. This model provides a sensitive and reliable platform for the study of optimizing flap physiology before, during, and after microvascular transfer.

Long-term composite tissue viability was demonstrated in the setting of immediate and delayed reperfusion. Those flaps subjected to 3 h of ischemia showed a greater insult secondary to relatively prolonged ischemia. At a cellular level, the effects of varied ischemic periods were demonstrated by

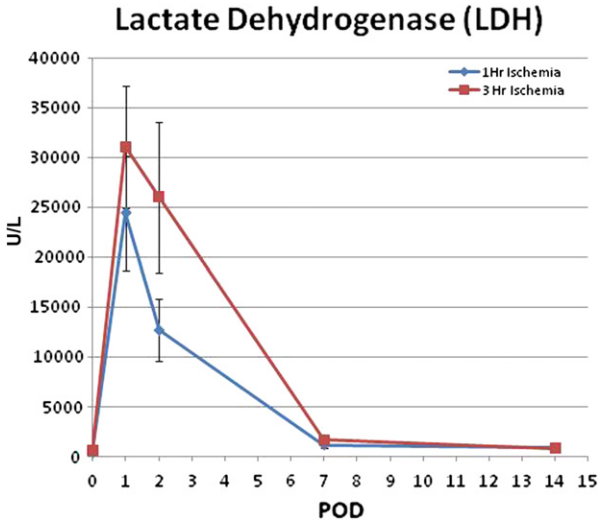

Fig. 10 – LDH levels during experimental course.

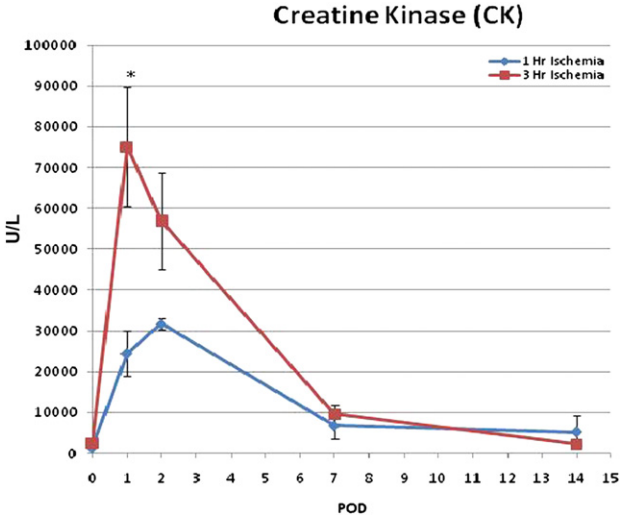

Fig. 11 – CK levels during experimental course. *P < 0.02.

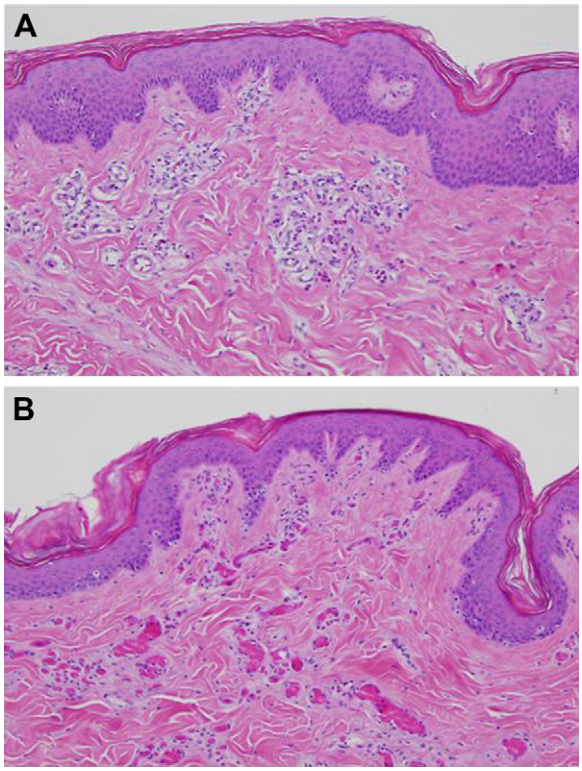

Fig. 12 – (A) Viable flap at 14 d. (B) Failed flap before 14 d showing areas of vascular congestion (arrows).

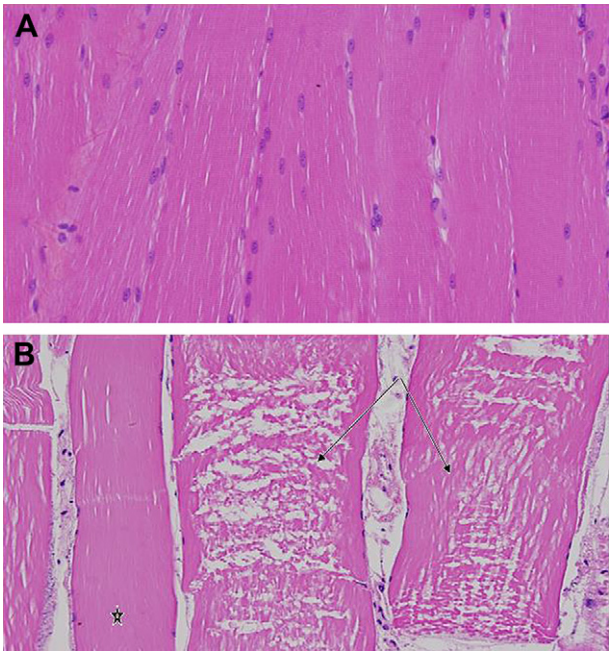

Fig. 13 – (A) 20× H&E stain (hematoxylin and eosin stain) longitudinal section. Viable flap. There are multiple longitudinal sections of muscle, with an increased number of satellite cells. Cross striations are clearly visible, likely a regenerative response. (B) 20× H&E longitudinal section. Nonviable flap. A relatively normal myocyte is represented by the star. The diameter of this myocyte is similar to those in (A). The arrows demonstrate myocytes that are swollen to at least two times normal, with fragmented sarcoplasm, cross striations that are no longer visible, and are separated by clear space (edema).

the disparity in the systemic levels of various biomarkers. All flaps subjected to ischemia exhibited a significant rise in biomarkers when compared with baseline. However, those flaps subjected to 3 h of ischemia had a more pronounced elevation in LDH, CK, and AST, with the difference in CK and AST elevations being statistically significant.

This large animal model serves as a novel system for the study of vascularized composite tissue flap physiology. The feasibility of auto- and allotransplantation of the gracilis myocutaneous flap in the swine is demonstrated. Flaps subjected to the stresses of various ischemic intervals can be evaluated at a cellular level using biomarkers that have been validated in the literature as markers for muscle injury and breakdown (LDH, CK, and AST).

Reperfusion injury poses significant limitations on the viability of transplanted tissues. The restoration of oxygen-rich blood to tissues that were previously ischemic leads to the damaging mechanisms of reperfusion injury, which include reactive oxygen species, complement system, and pro-inflammatory cytokines [9–11]. Reperfusion injury has wide clinical relevance and continued efforts have been aimed at therapeutic interventions. Unlike typical therapeutic interventions aimed at delayed attempts to ameliorate the ischemia-reperfusion process, a unique aspect of this model is the ability to pretreat tissue before ischemia. This has clinical relevance to reconstructive transplantation in general. The added ability to manipulate the physiologic milieu pre-injury may give additional insight into reperfusion injury mechanisms and counter-mechanisms. Inflammatory mediators, such as complement, prostaglandins, and other pro-inflammatory cytokines can be preemptively inhibited thus preventing deleterious overstimulation of the inflammatory process. Antioxidants can be prepositioned to ameliorate oxidative injury. Ultimately, preventative measures will be implemented to block the ischemia-reperfusion process before its onset through modalities, such as suspended animation using H_2S, warm *ex-vivo* perfusion, or advances in cold preservation [12,13]. Isolated *ex-vivo* control, as in this model, offers great latitude in treatment implementation.

A natural extension and overt objective of this line of research is translation of the model into allotransplantation. The composite tissue procurement and inset portion of the model are identical whether undergoing auto- or allotransplantation. The above principles designed to quell ischemia-reperfusion injury will be important adjuncts to the allotransplant model while suppressing excessive antigen release secondary to oxidative and inflammatory injury. In conjunction with implementation of principles learned in the ischemia-reperfusion studies, various immunomodulatory treatments will be studied and optimized in this large animal model. Standard triple drug immunosuppression will be compared with experimental protocols involving chimerism, co-stimulatory blockade, and other novel immunomodulatory regimens. Pursuit of

immunotolerance induction will be the ultimate intent of these studies. It is the progression toward preservation of protective immunity and solutions to current immunosuppressive drug toxicity that will unlock reconstructive allotransplantation as a safe mainstream treatment modality.

5. Summary

A novel method of vascularized gracilis myocutaneous flap transplantation was designed and validated in the Yorkshire swine as a platform for the study of optimizing postischemic flap physiology. Consistent flap survival during the 14-d survival period demonstrates the feasibility of this procedure. Systemic biomarker assays for skeletal muscle tissue injury (LDH, CK, and AST) showed ischemia period-dependent response thus providing assessment of ischemia-reperfusion injury at the cellular level. Follow-on studies will evaluate agents that mitigate ischemia-reperfusion injury and transition these findings to potentiate vascularized composite allotransplantation.

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